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# Recovery of Infectious Foot-and-Mouth Disease Virus from Suckling Mice after Direct Inoculation with In Vitro-Transcribed RNA

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**We assayed the infectivity of naked foot-and-mouth disease virus (FMDV) RNA by direct inoculation of suckling mice. Our results demonstrate that transcripts generated from full-length cDNA clones were infectious, as was virion-extracted RNA. Interestingly, infectious virus could be recovered from a mutant transcript encoding amino acid substitution L-147→P in capsid protein VP1, known to be noninfectious for BHK-21 cells. The model described here provides a useful tool for virulence studies in vivo, bypassing possible selection of variants during viral replication in cell culture.**

Foot-and-mouth disease virus (FMDV) is the causative agent of a highly contagious and economically important disease affecting wild and domestic cloven-hoofed animals (22, 43, 44). The virus belongs to the family *Picornaviridae* and consists of nonenveloped particles containing a positive-sense single-stranded RNA genome of about 8.5 kb. A unique open reading frame encodes all of the capsid and nonstructural viral proteins. FMDV virion RNA as well as full-length RNA transcripts derived from infectious cDNA clones have been widely reported to be infectious when transfected into susceptible cell lines (36, 51).

Because of the high mutation rates operating during genome replication, FMDV populations are genetically heterogeneous and exhibit an important potential for variation and adaptation (10, 11). Antigenic diversity of the FMDV populations has been widely described and still represents an important obstacle to disease control (10, 28). Other remarkable manifestations of the population dynamics of FMDV include modifications of receptor usage and host tropism (3, 17, 25), as well as the presence of a molecular memory that reflects the evolutionary history of the virus (9, 37).

In FMDV virions, the Arg-Gly-Asp (RGD) triplet located at the G-H loop of capsid protein VP1 (1, 23) is essential for the interaction with RGD-dependent integrins (6, 13, 18–20, 26, 29, 34) and with neutralizing antibodies (12, 15, 27, 48, 49). The RGD triplet is highly conserved among natural isolates of FMDV (11, 27), probably reflecting constraints imposed by the interaction with integrin receptors in vivo (13, 31). However, RGD can become dispensable upon large-population passages of FMDV in cell culture, which are associated with amino acid substitutions at the capsid surface and the use of alternative receptors for cell entry (3, 4, 16, 24, 38, 50).

Recent evidence suggests that changes in receptor specificity may also occur during FMDV replication in vivo. Unusual amino acid replacements affecting the RGD motif (R-141→G) or positions +1 and +4 relative to RGD (L-144→P and L-147→P), known to be critical for FMDV binding to some RGD-dependent integrins (29, 34), have been reported in vivo, in viruses escaping an immune response to synthetic peptides (45, 46), and in the process of adaptation of FMDV to guinea pigs (33). Moreover, engineered viruses based on a Chinese strain with a KGE sequence instead of RGD were able to cause disease in pigs (50).

The structural constraints imposed by FMDV interactions with RGD-dependent integrins in cell culture may have limited the identification of field isolates harboring amino acid alterations at key residues involved in receptor recognition. The noninfectious phenotype of viruses derived from an infectious cDNA clone harboring mutation L-147→P in the VP1 coding region was consistent with the inability of this virus to interact with integrin receptor molecules expressed in BHK-21 cells (33).

Because of limitations of receptor usage due to amino acid replacements on the FMDV capsid, it would be desirable to have an in vivo system to test the infectivity of viral RNA that would avoid a possible selection of FMDV variants during virus growth in cell culture.

FMDV is unable to productively infect adult mice, but during the first weeks of life, mice are susceptible to intraperitoneal inoculation of viral particles, which induces rapid disease and death (41). In the present report, we describe the infection and death of suckling mice by using naked viral RNA. During virus inactivation experiments with purified virus, Mussgay had previously shown that type $O_2$ FMDV RNA was infectious for mice by intracerebral inoculation (2, 30). Our results show that both virion-extracted RNA and RNA transcribed from full-length cDNA clones, including mutants unable to propagate in tissue culture, are infectious after direct transfection of suckling mice. The model offers a novel approach to assess the virulence of FMDV RNAs in vivo.

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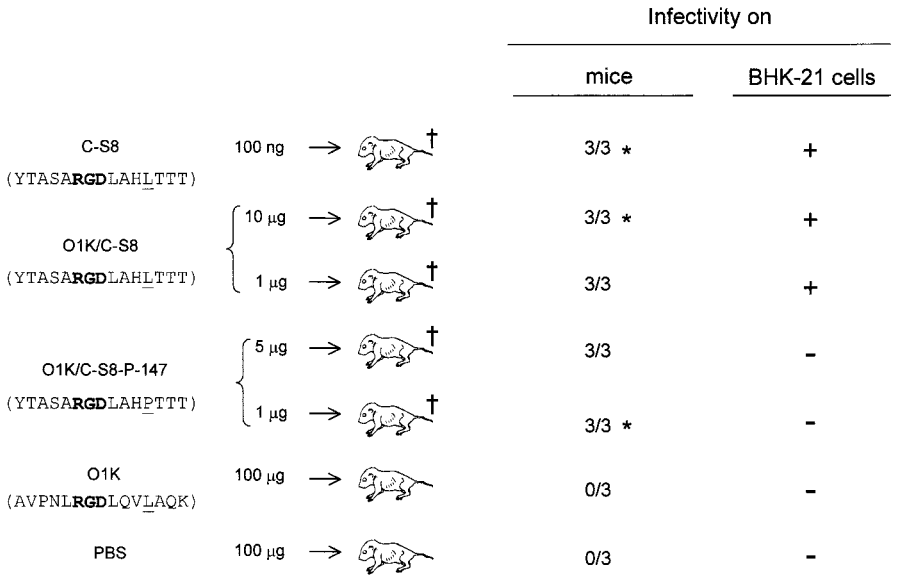


FIG. 1. Infectivity in mice and cell culture of extracts from RNA-inoculated mice. Homogenates from animals inoculated with FMDV genomic RNA (C-S8), RNA transcripts (O1K/C-S8, O1K/C-S8-P-147, and O1K), or PBS were used to inoculate suckling mice and BHK-21 cells. The ratio of dead versus inoculated animals is shown for each inoculum. + and −, recovery and no recovery, respectively, of infectious virus from BHK-21 cells by CPE induction up to 48 h p.i. with homogenates. Asterisks represent the groups of animals used for sequence analysis. The amino acid sequence at the G-H loop of VP1 is indicated below each RNA. The residue at position 147 of VP1 is underlined. The RGD triplet is indicated in boldface.

**Virulence of FMDV RNAs after direct inoculation of suckling mice.** In order to evaluate their infectivity in vivo, virion-extracted FMDV C-S8c1 RNA and transcripts from different infectious clones were inoculated into suckling mice. C-S8c1 is a derivative of a swine isolate from Spain (42).

Chimeric O1K/C-S8 transcripts were obtained from plasmid pO1K/C-S8c1 (5), which carries the type C FMDV region from nucleotides (nt) 1739 to 4066 (corresponding to amino acids S-33 of VP4 to K-62 of 2B) in a full-length FMDV O1K infectious clone (51). Chimeric O1K/C-S8-P-147 transcripts were derived from plasmid pO1K/C-S8c1-VP1 (33) and carry a single mutation encoding substitution L-147→P within the G-H loop of capsid protein VP1; this replacement is the only difference from pO1K/C-S8c1 (Fig. 1). O1K transcripts were derived from plasmid pDM (40), constructed by using the FMDV O1K infectious clone (51), and carry engineered restriction sites flanking the 3′ noncoding region with no effect on in vitro and in vivo infectivity (40).

All transcripts were generated by using SP6 RNA polymerase (Promega, Madison, Wis.), and plasmids were linearized with *Hpa*I (New England Biolabs, Beverly, Mass.) as a template. After transcription, reaction mixtures were treated with RQ1 DNase (1 U/μg of RNA; Promega). RNAs were extracted with phenol-chloroform and precipitated with ethanol. RNA integrity and concentration were determined by agarose gel electrophoresis.

Groups of mice about 7 days old were inoculated intraperitoneally with 100 μl of different amounts of the viral RNAs described above diluted in phosphate-buffered saline (PBS) containing 20 μg of Lipofectin (Gibco BRL, Rockville, Md.). Dead animals were scored up to 9 days after inoculation, and survivors were euthanized. In all experiments, control animals were mock inoculated with PBS alone and with PBS and Lipofectin according to the same procedure (Table 1).

Genomic RNA purified from FMDV C-S8 virions caused death in mice, and the animals manifested symptoms (tremors, ataxia, and paralysis of the hind limbs) similar to those observed prior to death in animals inoculated with infectious virus. Twenty nanograms of C-S8 RNA caused the death of approximately 50% of the animals inoculated.

Both O1K/C-S8 and O1K/C-S8-P-147 RNA transcripts were virulent for suckling mice, inducing symptoms and death with about 5- to 25-fold-lower killing efficiency than C-S8 genomic RNA, respectively (Table 1). Remarkably, the presence of VP1 P-147 in O1K/C-S8-P-147 was only responsible for an approximately fivefold reduction in lethality to mice compared to O1K/C-S8 (L-147). However, O1K/C-S8-P-147 RNAs failed to induce productive infection after transfection of BHK-21 cells, with L-147 revertants the only progeny recovered at late times postelectroporation (33). The amounts of RNA needed to cause the death of 50% of the inoculated animals were 100 to 200 ng for O1K/C-S8 transcripts and approximately 500 ng for O1K/C-S8-P-147 transcripts. The time required for mouse death ranged from 3 to 7 days postinoculation (p.i.), depending on the RNA concentration and experiment (data not shown). O1K transcripts failed to kill any mice at RNA amounts as large as 100 μg at day 9 p.i., in contrast to their high degree of infectivity when transfected on BHK-21 cells (Table 2). None of the mice inoculated with PBS containing Lipofectin or with PBS alone died in any experiment, suggesting that viral RNAs were the only cause of death. Moreover, three animals were inoculated with 100 μg of tRNA, none of whom died or showed any sign of disease (data not shown). The fact that the highest doses of RNA assayed (O1K transcripts) did not kill

TABLE 1. Infectivity of FMDV RNAs in suckling mice

| Amt of RNA[a] | No. of dead mice/no. inoculated |
|---|---|
| C-S8 virion | |
| 500 ng | 1/1 |
| 250 ng | 2/2 |
| 100 ng | 8/9 |
| 50 ng | 6/8 |
| 20 ng | 3/7 |
| 10 ng | 2/7 |
| 1 ng | 1/7 |
| In vitro transcripts | |
| O1K/C-S8 | |
| 50 μg | 1/1 |
| 25 μg | 2/2 |
| 10 μg | 1/2 |
| 5 μg | 2/2 |
| 1 μg | 7/7[b] |
| 500 ng | 5/7 |
| 200 ng | 5/7 |
| 100 ng | 2/7 |
| 50 ng | 0/7 |
| 20 ng | 0/7 |
| 10 ng | 0/5 |
| O1K/C-S8-P-147 | |
| 5 μg | 1/2[b] |
| 1 μg | 5/6[b] |
| 500 ng | 3/7 |
| 200 ng | 1/7[b] |
| 100 ng | 1/7 |
| 50 ng | 0/7 |
| 20 ng | 0/7 |
| 10 ng | 0/5 |
| O1K | |
| 100 μg | 0/2 |
| 50 μg | 0/4 |
| 25 μg | 0/4 |
| 10 μg | 0/2 |

[a] Amount of RNA used for intraperitoneal inoculation.
[b] Group of mice used for sequence analysis.

any animal confirms that RNA toxicity is not to be considered as a cause of death but represents the specific infectivity of each RNA.

The data shown in Table 2 highlight the difference in virulence of the FMDV-derived RNAs when infectivity was estimated by RNA inoculation of suckling mice, in contrast with data obtained from cultured cells after transfection of RNAs or even with viruses recovered from transfected cells for mice inoculation. While C-S8 virion RNA and O1K/C-S8 transcripts were infectious when transfected into susceptible cell lines, and the viruses recovered from transfections induced death in mice at high dilutions (Table 2), O1K/C-S8-P-147 transcripts were unable to induce cytopathic effect (CPE) in transfected cells. However, direct inoculation of this mutant RNA efficiently induced death in mice (Table 1), suggesting a role for L-147 in receptor interaction with BHK-21 but not for viral entry in murine cells in vivo. Expression of $\alpha_v\beta_3$ integrin is reported to be down-regulated in striated muscle tissue during development (7), suggesting an effect on the susceptibility of newborn mice to viruses using this receptor, depending on the age of the animals at the time of inoculation (32). It has also been reported that productive coxsackievirus A13 infection of muscle cells cultured from tissues of fetal mice is limited to the stage of differentiation (14).

TABLE 2. Infectivity of viral RNAs on cell culture and virulence of the resultant viruses in suckling mice

| RNA | Infectivity on BHK-21 cells (PFU/μg)[a] | Virulence in suckling mice ($LD_{50}$/ml)[b] |
|---|---|---|
| C-S8 | $5 \times 10^4$ | $2 \times 10^{-7}$ |
| O1K/C-S8 | $1.3 \times 10^3$ | $1 \times 10^{-7}$ |
| O1K/C-S8-P-147 | <1 | |
| O1K | $4.7 \times 10^3$ | $1 \times 10^{-3}$ |

[a] RNAs were transfected into BHK-21 cells with Lipofectin (40) and incubated at 37°C under a 0.6% agar overlay. Monolayers were fixed and stained 48 h following transfection.
[b] Viruses recovered after transfection of the indicated RNA and two additional passages on BHK-21 cells were used for intraperitoneal inoculation of suckling mice. The titer of the viral inocula was $10^7$ PFU/ml. Fifty percent lethal doses ($LD_{50}$s) were determined as described previously (35).

The lack of virulence of O1K transcripts in mice is likely related to the presence of an Arg residue at position 56 of VP3. For type O FMDV, the presence of a highly charged Arg residue at that position has been associated with adaptation to cell culture (21) and is accompanied by a $10^5$-fold attenuation in cattle, increased affinity for heparin, and improved replication in CHO cells (38). The presence of R-56 in the VP3 coding region, the high affinity of binding to heparin of the viral progeny obtained from pO1K in BHK-21 cells, and growth in CHO cells were experimentally confirmed for the O1K parental clone by sequencing, heparin-Sepharose binding assays, and in vitro infection experiments, respectively (data not shown).

Viruses recovered after transfection of BHK-21 cells with O1K transcripts were attenuated 4 logs in suckling mice compared to cell culture infectivity (Table 2). Accordingly, O1K transcripts were attenuated at least 3 logs in suckling mice, compared to O1K/C-S8 transcripts, and we did not reach the minimum levels of RNA required for lethality.

**Homogenates from dead mice contain infectious virus maintaining the original capsid sequence after passage.** To confirm that infectious virus had indeed been generated following RNA inoculation in suckling mice, crude extracts from these animals were assayed for infectivity in mice as well as in cell culture. Small pieces of brain and hind limbs of frozen dead or euthanized animals (about 0.3 to 0.5 g), previously inoculated with different amounts and species of RNA, were homogenized in 1 ml of PBS, clarified by centrifugation, filtered through 0.45-μm-pore-diameter filters, and then used for inoculation of BHK-21 cells and injection of suckling mice (Fig. 1). Dilutions of the homogenates in PBS up to $10^{-2}$ were inoculated into suckling mice by the same procedure described for RNA primary inoculation. Infectivity could be transmitted to mice in all cases in which primary RNA inoculation had caused death (C-S8 virion RNA and O1K/C-S8 and O1K/C-S8-P-147 transcripts) by using a $10^{-2}$ dilution of the corresponding extracts. All animals inoculated with those homogenates were found dead at day 3 p.i. In the case of mice inoculated with O1K homogenate, none of the three animals showed any sign of disease, like those injected with extracts from mock-inoculated animals, when $10^{-2}$ and $10^{-1}$ dilutions or undiluted extracts were used. The ability to cause infection and death by using the mouse homogenates as an inoculum strongly suggested the presence of infectious viral particles. To

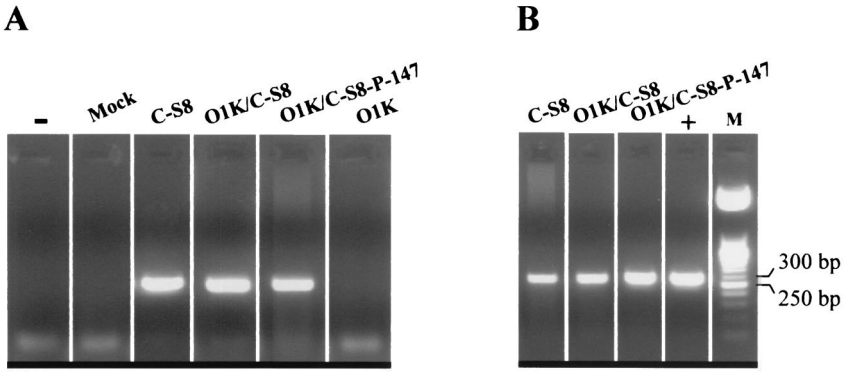


FIG. 2. Detection of FMDV RNA in mouse extracts. RT-PCR with primers A and B (39) was carried out on RNAs extracted from mouse homogenates. Fifty percent of each reaction mixture was loaded on a 2% agarose gel. The product of the expected size is a 290-bp fragment spanning a conserved region in the 3Dpol gene. Panel A shows the results for primary inoculation of the RNAs indicated at the top. Amplification products for mice inoculated with homogenates from mice inoculated with the RNAs indicated at the top are shown in panel B. + and −, RNA-positive (FMDV CS-8 RNA) and RNA-negative (in the absence of RNA) control reactions, respectively. M, molecular weight marker XIII (Roche, Mannheim, Germany).

prove the resistance of the infectious capacity in these homogenates to RNase treatment, a 1/5 dilution of homogenate from a mouse dead after inoculation with 10 μg of O1K/C-S8 transcripts was incubated in the presence or absence of 7.5 μg of RNase A for 15 min at room temperature. Following incubation, two animals were inoculated with the RNase-treated fractions, and two were inoculated with untreated samples. As a control, two animals were inoculated with PBS containing 7.5 μg of RNase A. Only the two control animals survived (data not shown).

When the homogenates from RNA-inoculated mice were tested for their infectivity on BHK-21 cells (Fig. 1), induction of CPE could be observed for animals inoculated with C-S8 genomic RNA and O1K/C-S8 transcripts. Homogenates from mice inoculated with O1K/C-S8-P-147 transcripts failed to produce CPE on BHK-21 cells (three trials), as expected from the absence of infectivity of FMDV mutants harboring VP1 substitution L-147→P in cell culture (33). Similarly, no CPE was observed in cells inoculated with homogenates from mice injected with O1K transcripts.

The presence of FMDV RNA in mouse homogenates from dead animals was analyzed by reverse transcription-PCR (RT-PCR) analysis using both animals primarily inoculated with RNA and mice inoculated with homogenates, processed as described above. The primers used amplify a 290-bp fragment in the 3Dpol gene of all seven FMDV serotypes (39). As shown in Fig. 2, we were able to detect viral RNA in mice dead after inoculation with RNA or homogenates corresponding to C-S8 genomic RNA, as well as in mice inoculated with O1K/C-S8 and O1K/C-S8-P-147 transcripts. No amplification product could be observed in samples from animals inoculated with O1K transcripts or PBS. Thus, the presence of viral RNA correlated with infectivity and death in mice.

In order to study the stabilities of viral capsid sequences in mice and compare them with those present in the primarily inoculated RNA, nucleotide sequences of VP2, VP3, and VP1 coding regions were determined for representative animals of each group, including mice inoculated with RNA and those inoculated with tissue homogenates (Table 1 and Fig. 1). Total RNA was extracted from mouse homogenates by the guanidinium isothiocyanate method (8). Using primers SB6 (CTCC ACATCTCCAGCCAACTTGAGCA; reverse of C-S8c1 nt 3845 to 3870) and SB5 (ACCTCTACACACACAACCAACA CCC; C-S8c1 nt 1804 to 1828), a 2,067-bp cDNA fragment spanning the genes coding for capsids VP2 through VP1 was amplified by RT-PCR. Internal primers used for sequencing have been described previously (47). Consensus sequences were determined with an ABI 373 (Applied Biosystems) automated sequencer. The sequence of RNA extracted from animals inoculated with C-S8, O1K/C-S8, and O1K/C-S8-P-147 confirmed the presence in progeny RNA of the original sequence in the G-H loop of VP1 and the capsid region, respectively (Fig. 1). RNAs extracted from three different mice inoculated with O1K/C-S8-P-147 RNA in two independent experiments and a single mouse inoculated with the corresponding homogenate were included in the sequencing reactions (see Table 1 and Fig. 1, respectively). In all cases, a C residue was present at position 3647, leading to an L-147→P change in VP1, proving that the mutation present in O1K/C-S8-P-147 RNA is conserved after passage in vivo. Only one of the three animals inoculated with O1K/C-S8-P-147 transcripts showed an additional mutation in the capsid VP3 gene at position 2675 (C→T leading to replacement T-42→I) compared to the parental clone, O1K/C-S8. For C-S8 (one RNA-inoculated mouse) and O1K/C-S8 (two mice inoculated with RNA and homogenate, respectively), no sequence changes were detected in the region analyzed compared to their respective inoculated RNAs.

The results presented here provide evidence that either naked virion FMDV RNA or transcripts generated from full-length cDNA clones are able to initiate infection and kill suckling mice after direct inoculation. Moreover, infectious viral progeny could be recovered from dead animals, and these populations exhibited infectivity phenotypes resembling those of the original RNAs used for inoculation either in vitro or in vivo. Inoculation of genetically modified RNAs in suckling mice as we describe here is a novel infectivity assay that allows assessment of the virulence of FMDV variants in vivo, includ-

ing viruses unable to propagate in cell culture and thus bypassing in vitro selection of sequence variants. Additionally, the assay allows recovery of virus mutants that might be selected against or even unable to productively infect cultured cell lines. Alternative methods to generate viruses that are unable to bind BHK-21 cell receptors, like high-efficiency electroporation and first-cycle virus injection into mice, could be considered. However, this method can lead to the selection and subsequent replication of sequence revertants, generating undesired viral diversity. Direct inoculation of RNA ensures sequence homogeneity and circumvents additional steps of cell culture. FMDV RNA in vivo transfection opens up a wide range of applications for identification and characterization of virulence determinants, host interactions, and viral tropism studies.

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